Inventors: Xiaolie Yu
Milorad Susa
Cornelius Knabbe
Rolf Schmidt
Till T. Bachman

METHOD AND DEVICE FOR DETECTING QUINOLONE-RESISTANT *ESCHERICHIA COLI*

FIELD OF THE INVENTION

[0001] The present invention pertains to a method for detecting quinolone-resistant *Escherichia coli* strains in a biological sample material. The present invention also relates to a kit adapted to perform the present method.

BACKGROUND OF INVENTION

[0002] Bacterial infections are generally treated with antibiotics, among which quinolones have proven to be one of the most highly potent agents for use in human. In the past, fluoroquinolones have been widely used as broad spectrum antimicrobial agents in clinical medicine with the result that bacteria have developed resistance against this agent.

[0003] One of the most concerned species of bacteria to be treated with quinolones is *E. coli*, which causes a number of infections, primarily in and around artificial or natural openings of the body, such as lesions in the skin or the urinary tract. Particularly, experience in and information about the treatment of urinary tract infections shows that 90 % of the antibiotics administered are quinolones, while in the meantime about 8 % of the *E. coli* strains have become resistant. Therefore, the ordinary regimen does not apply in a number of cases, which the attending physician will normally recognize only at a later stage of the infection/bacterial growth, with a concurrent destruction of the infested tissue. In addition, quinolone-resistant *E. coli* may also prove to be a potential threat to neutropenic patients with leukemia, who receive a quinolone as prophylaxis.

[0004] In general, the therapeutic or prophylactic use of quinolones without considering possible resistance of the infecting pathogen may lead to treatment failures as well as to an induction of new resistances.

[0005] Therefore, there is a need in the art to get information about potential resistances occurring in the bacterial population to be treated.

[0006] Up to now the standard methods to determine an antibiotic resistance are based on phenotypic identification, which is time consuming and is in certain cases not sensitive and precise enough.

[0007] An approach in the art to cope with these problems focuses on the investigation of polypeptides accounting for the quinolone resistance in pathogenic bacteria. Several analyses have been developed in order to gain such information, for example a single-stranded conformational polymorphism (SSCP) analysis (Ouabdesselam S, Hooper DC, Tankovic J, Soussy CJ, Antimicrobial Agents and Chemotherapy **39** (1995), 1667-70), a mismatch amplification mutation assay (MAMA; Qiang YZ, Qin T, Fu W, Cheng WP, Li YS, Yi G., J Antimicrob Chemother **49** (2002), 549-52) and a restriction fragment length polymorphism (RFLP) analysis (Hooper DC, Wolfson JS, Ng EY, Swartz MN., Am J Med **82** (1987), 12-20).

[0008] However, all the above methods and assays exhibit a variety of different shortcomings. In particular, with a SSCP only the region of mutation may be detected, but not the exact position of mutation. With the MAMA procedure, only one variant may be determined at a time, or else a cost and work intensive multiplex PCR has to be performed. RFLP detects only the position of the mutation, but not the substitution. In addition, none of the methods accurately predicts whether the bacterial sample exhibits resistance to the agents utilized.

[0009] Therefore, a need exists to rapidly and reliably detect the presence of resistant strains of bacteria. Furthermore, such a detection assay should process multiple samples simultaneously and inexpensively.

SUMMARY OF THE INVENTION

[0010] It is, therefore, one object of the present invention to provide a method for detecting the presence of quinolone resistant *E. coli* strains in a biological sample.

[0011] It is also an object of the present invention to provide micro-arrays and kits for use in detecting the presence of quinolone resistant *E. coli* strains in a biological sample.

[0012] In accomplishing these and other objects of the invention, there is provided, in accordance with one aspect of the invention a method for detecting the presence of quinolone resistant *E. coli* strains in a biological sample, which method comprises the steps (i) obtaining DNA from a biological sample, (ii) optionally amplifying the DNA contained in the sample with primers specific for the target sequence, (iii) contacting the DNA contained in the biological sample or obtained in step (ii) with a micro-array comprising at specific pre-determined locations of the array two sets of capture probes, which are derived from the sequence of a gyrA gene of

E.coli, and comprise the sequence R_1-(X)-R_2, wherein (a) X designates all permutations of the triplet at amino acid position 83 and 87 of the gyrA polypeptide of *E.coli,* and wherein (b) R_1 and R_2 are sequences derived from the gyrA gene of *E.coli* adjacent to the triplet of either position 83 or 87 of the gyrA polypeptide and comprising of from about 5 to 20 nucleotides, under conditions allowing hybridization of complementary strands, and (iv) determining, at which location on the array binding occurs, wherein a change in the nucleic acid at the said positions resulting in a change of an amino acid is indicative of the development of a resistance against quinolones. In one embodiment, the change in the nucleic acid sequence results in an amino acid change of the gyrA polypeptide to leucine at position 83 and/or asparagine or tyrosine at position 87.

[0013] The invention also provides a micro-array containing at specific predetermined locations of the array two sets of capture probes, derived from the sequence of a gyrA gene of *E.coli*, comprising the sequence R_1-(X)-R_2, wherein (a) X designates all permutations of the triplet at amino acid position 83 and 87 of the gyrA polypeptide of *E.coli* and (b) R_1 and R_2 are sequences derived from the gyrA gene of *E.coli* adjacent to the triplet of either position 83 or 87 of the gyrA polypeptide and comprising of from about 5 to 20 nucleotides.

[0014] In another embodiment, there is provided a kit for detecting the presence or absence of a quinolone resistant *E. coli* strain in a biological sample, containing a micro-array containing at specific predetermined locations of the array two sets of capture probes, derived from the sequence of a gyrA gene of *E.coli*, comprising the sequence R_1-(X)-R_2, wherein (a) X designates all permutations of the triplet at amino acid position 83 and 87 of the gyrA polypeptide of *E.coli* and (b) R_1 and R_2 are sequences derived from the gyrA gene of *E.coli* adjacent to the triplet of either position 83 or 87 of the gyrA polypeptide and comprising of from about 5 to 20 nucleotides, and optionally buffers and reagents.

[0015] Other objects, features and advantages of the present invention will become apparent from the following detailed description. The detailed description and specific examples, while indicating preferred embodiments, are given for illustration only since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description. Further, the examples demonstrate the principle of the invention and cannot be expected to specifically illustrate the application of this invention to all the examples where it will be obviously useful to those skilled in the prior art.

BRIEF DESCRIPTION OF THE FIGURES

[0016] Figures 1 A- D show the results of a hybridization of clinical isolates with labeled target DNA on a micro-array.

DETAILED DESCRIPTION OF THE INVENTION

[0017] In the studies leading to the present invention a number of clinical isolates of *E. coli* known to be quinolone resistant have been investigated, while it has been surprisingly noted that in contrast to the quinolone sensitive strain, all of the resistant strains exhibited mutations in the gyrA polypeptide in at least one of amino acid positions 83 and 87. This focus on these two amino acid positions in resistant strains has been confirmed by additional studies so that the present invention is essentially based on the finding that in order to detect a quinolone resistance in *E.coli*, it is sufficient to provide data about these two positions in the gyrA polypeptide of *E.coli*, only.

[0018] Without wishing to be bound to any theory, it is presently believed that even though these two positions are not the sole mutations occurring in the gyrA polypeptide of quinolone resistant strains, they seem to be mainly involved in the development of resistance due to a folding of the resulting polypeptide preventing interaction with quinolones.

[0019] Another gene of interest that conveys quinolone resistance is topoisomerase IV. Of particular interest is subunit A, which is encoded by the parC gene. In this gene, three amino acid positions, 80, 84 and 87, are proposed as locations for the detection of quinolone resistance.

Definitions

[0020] In the present description the following definitions apply:

[0021] The terms "micro-array" and "array of oligonucleotides", which are used interchangeably in the present invention, refer to a multiplicity of different nucleotide sequences attached or positioned on one or more solid supports where, when there is a multiplicity of supports, each support bears a multiplicity of nucleotide sequences. Both terms may refer to the entire collection of nucleotides on the support(s) or to a subset thereof. In one embodiment, the nucleotide sequence is attached through a single terminal covalent bond. The support is generally composed of a solid surface which may be selected from the group consisting of glasses, electronic devices, silicon supports, silica, metal or mixtures thereof prepared in format selected from the group of slides, discs, gel layers and/or beads.

[0022] As used in present invention, the term "probe" or "capture probe" in the sense of the present invention is defined as a nucleotide sequence representing specific parts of the gyrA gene or parC gene, respectively, of *E.coli* covering amino acid positions 83 and 87 (gyrA) or 80, 84 or 87 of parC, respectively. The sequences have different lengths, e.g. between about 10 and 43 nucleotides, and are either chemically synthesized in situ on the surface of the support or laid down thereon. They are capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a nucleotide probe may include natural (i.e. A, G, C, or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in an oligonucleotide probe may be joined by a linkage other than a phosphodiester bond, such as *e.g.* peptide bonds, so long as it does not interfere with hybridization.

[0023] The term "target nucleic acid" refers to a nucleic acid, to which the nucleotide probe specifically hybridizes.

[0024] The term "gyrA gene" as used in the present application comprises the gyrA gene of *E.coli* and its variants due to mutations and changes in different strains.

[0025] The term "parC gene" as used in the present application comprises the parC gene sequence of *E.coli* and its variants due to mutations and changes in different strains.

[0026] Th term "nucleotide sequence" as used herein refers to oligonucleotide(s), polynucleotide(s) and the like including analogous species wherein the sugar-phosphate backbone is modified and/or replaced, provided that its hybridization properties are not destroyed.

[0027] The phrase "hybridizing specifically to" refers to the binding, duplexing or hybridizing of a molecule substantially to or only to a particular nucleotide sequence or sequences under stringent conditions when that sequence is present in a complex mixture of DNA or fragments thereof.

[0028] The terms "background" or "background signal intensity" refers to hybridization signals resulting from non-specific binding, or other interactions, between the labeled target nucleic acids and components of the nucleotide array (e.g., the nucleotide probes, control probes, the array substrate, etc.).

Description

[0029] In order to perform the present method, a DNA from a biological sample is obtained in a first step from an individual to be treated or deemed to harbor a resistant strain. The biological sample/material may be any material supposed to contain a pathogenic *E.coli*, such as tissue from an area of a lesion, blood, or body secretions, such as sputum or urine. For some applications, it may be appropriate to transfer the biological sample into a medium suitable for the growth of *E.coli*, *e.g.* on LB agar plates.

[0030] The DNA contained in the biological sample may liberated from the *E.coli* cells or isolated according to techniques well known in the art, *e.g.* via QIAprep® Spin Miniprep Kit protocol (Qiagen, Hilden, Germany). Alternative appropriate methods for obtaining DNA may be chosen, depending on the specific starting material. Such an isolation step assists in preventing the development of extensive background signals during the hybridization step, in case no other selection step is applied.

[0031] In one embodiment, the DNA contained in the biological sample or isolated therefrom may be amplified via a polymerase chain reaction (PCR) using one or more primers, which provides the advantage of augmenting the specific material to be investigated only and also to incorporate a selection step. In case of using one primer only, the complementary strand of the DNA of interest will be synthesized. Alternatively, at least two primers are utilized, allowing an exponential amplification of the material to be investigated. According to an alternative embodiment a nested PCR is carried out, wherein 2 pairs of primers are put to use. A first set of primers is selected to amplify a sequence largely around the target sequence. Then, the second pair of primers are used to amplify a sequence lying within the sequence amplified first. Proceeding accordingly gives the inherent advantage that a second selection step is incorporated in the present method, which assists in reducing the background. After the completion of the PCR reaction, the PCR product may be purified if desired.

[0032] When using an amplification step, the DNA may at the same time be labeled, *e.g.* by including in the amplification process nucleotides harboring an appropriate label. Alternatively, a label may be attached to the nucleic acid, including, for example, nick translation or end-labeling by attachment of a nucleic acid linker joining the sample nucleic acid to a label.

[0033] Detectable labels suitable for use in the present invention include any composition detectable by spectroscopic, photochemical, biochemical, immunochemical, electrical, optical or

chemical means. Useful labels in the present invention include biotin for staining with labeled streptavidin conjugate, magnetic beads (e.g., Dynabeads™), fluorescent dyes (e.g., cyanine dyes, such as Cy5, fluorescein, texas red, rhodamine, green fluorescent protein, and the like), radiolabels (e.g., ^{3}H, ^{125}I, ^{35}S, ^{14}C, or ^{32}P), enzymes (e.g., horse radish peroxidase, alkaline phosphatase and others commonly used in an ELISA), and colorimetric labels such as colloidal gold or colored glass or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. Patents teaching the use of such labels include U.S. Patent Nos. 3,817,837; 3,850,752; 3,939,350; 3,996,345; 4,277,437; 4,275,149; and 4,366,241.

[0034] In order to allow detection of the presence of a single mutation, the target- or probe-DNA should not be too long, since otherwise renaturation in solution, or base-pairing with a capture probe allowing mismatches may occur. The desired length of such a probe DNA should be of from about 10 to 50 nucleotides, preferably 15 to 40, more preferably 15 - 30, even more preferred 15- 25 nucleotides and may be obtained by either selecting the primers during the amplification step accordingly, or by fragmenting the DNA put to use after an amplification step.

[0035] The target-/probe-DNA thus obtained is then contacted with the capture probes on the micro-array under conditions allowing hybridization of complementary strands only. In general, since a difference in at least one nucleotide is studied under certain conditions, stringent hybridization conditions are selected, *e.g.* adjusting the hybridization temperature to be about 1° - 5°C below the calculated thermal melting point (T_m) of a the specific sequence at a defined ionic strength and pH. The T_m is the temperature (at the defined ionic strength, pH) at which 50% of the probes complementary to the target sequence hybridize to the target sequence at equilibrium. Another possibility to adjust stringent conditions resides in adding destabilizing agents, such as e.g. formamide.

[0036] In principle, the capture probes on the micro-array comprise the sequence R_1-(X)-R_2 and are provided in two sets on the array. In one set, the sequences R_1 and R_2, which may be of a length of from about 5 to about 20 nucleotides each, are derived from the sequences of the gyrA gene of *E.coli* adjacent to the triplet encoding the amino acid at position 83 in the gyrA polypeptide, while in the second set of capture probes the sequences R_1 and R_2, which may exhibit the same length as indicated above, are derived from the sequence of the gyrA gene of *E.coli* adjacent to the triplet encoding the amino acid at position 87 in the gyrA polypeptide.

[0037] In a preferred embodiment the sequences R_1 and R_2 are designed such that known mutations of the gene encoding the gyrA polypeptide around positions 83 and 87, *e.g.* at positions 85 and 89, are taken into account. Hence, the positions 85 and 89 may also be permutated to cover all potential exchanges at these positions and permit an extremely accurate means to determine a SNP at positions 83 and 87, respectively.

[0038] An exemplary set of capture probes is shown in table I below.

Table I

Name	Position	Variation	Sequence (3' → 5')	Amin Acid
E.coli_GyA83A1	83	85(GTC)	AT GGT GAC TAG GCG GTC TA	Stop code
E.coli_GyA83T1	83	85(GTC)	AT GGT GAC TTG GCG GTC TA	Leu
E.coli_GyA83G1	83	85(GTC)	AT GGT GAC TGG GCG GTC TA	Trp
E.coli_GyA83C1	83	85(GTC)	AT GGT GAC TCG GCG GTC TA	Ser
E.coli_GyA83A2	83	85(GTT)	AT GGT GAC TAG GCG GTT TA	Stop code
E.coli_GyA83T2	83	85(GTT)	AT GGT GAC TTG GCG GTT TA	Leu
E.coli_GyA83G2	83	85(GTT)	AT GGT GAC TGG GCG GTT TA	Trp
E.coli_GyA83C2	83	85(GTT)	AT GGT GAC TCG GCG GTT TA	Ser
E.coli_GyA83AU	83	85(GTI)	AT GGT GAC TAG GCG GTI TA	Stop code
E.coli_GyA83TU	83	85(GTI)	AT GGT GAC TTG GCG GTI TA	Leu
E.coli_GyA83GU	83	85(GTI)	AT GGT GAC TGG GCG GTI TA	Trp
E.coli_GyA83CU	83	85(GTI)	AT GGT GAC TCG GCG GTI TA	Ser
E.coli_GyA87A1	87	85(GTC)/89(ATT)	GCG GTC TAT AAC ACG ATT G	Asn
E.coli_GyA87T1	87	85(GTC)/89(ATT)	GCG GTC TAT TAC ACG ATT G	Tyr
E.coli_GyA87G1	87	85(GTC)/89(ATT)	GCG GTC TAT GAC ACG ATT G	Asp
E.coli_GyA87C1	87	85(GTC)/89(ATT)	GCG GTC TAT CAC ACG ATT G	His
E.coli_GyA87A2	87	85(GTT)/89(ATT)	GCG GTT TAT AAC ACG ATT G	Asn
E.coli_GyA87T2	87	85(GTT)/89(ATT)	GCG GTT TAT TAC ACG ATT G	Tyr
E.coli_GyA87G2	87	85(GTT)/89(ATT)	GCG GTT TAT GAC ACG ATT G	Asp
E.coli_GyA87C2	87	85(GTT)/89(ATT)	GCG GTT TAT CAC ACG ATT G	His
E.coli_GyA87A3	87	85(GTC)/89(ATC)	GCG GTC TAT AAC ACG ATC G	Asn
E.coli_GyA87T3	87	85(GTC)/89(ATC)	GCG GTC TAT TAC ACG ATC G	Tyr
E.coli_GyA87G3	87	85(GTC)/89(ATC)	GCG GTC TAT GAC ACG ATC G	Asp
E.coli_GyA87C3	87	85(GTC)/89(ATC)	GCG GTC TAT CAC ACG ATC G	His
E.coli_GyA87A4	87	85(GTC)/89(ATT)	GCG GTT TAT AAC ACG ATC G	Asn
E.coli_GyA87T4	87	85(GTC)/89(ATT)	GCG GTT TAT TAC ACG ATC G	Tyr
E.coli_GyA87G4	87	85(GTC)/89(ATT)	GCG GTT TAT GAC ACG ATC G	Asp
E.coli_GyA87C4	87	85(GTC)/89(ATT)	GCG GTT TAT CAC ACG ATC G	His
E.coli_GyA87AU1	87	85(GTI)/89(ATI)	GCG GTI TAT AAC ACG ATI G	Asn
E.coli_GyA87TU1	87	85(GTI)/89(ATI)	GCG GTI TAT TAC ACG ATI G	Tyr
E.coli_GyA87GU1	87	85(GTI)/89(ATI)	GCG GTI TAT GAC ACG ATI G	Asp
E.coli_GyA87CU1	87	85(GTI)/89(ATI)	GCG GTI TAT CAC ACG ATI G	His

E.coli_GyA87A5	87	85(GTC)/89(ATT)	GCG GT**C** TAT **GA**C ACG AT**T** G	Asn
E.coli_GyA87T5	87	85(GTC)/89(ATT)	GCG GT**C** TAT **GT**C ACG AT**T** G	Tyr
E.coli_GyA87G5	87	85(GTC)/89(ATT)	GCG GT**C** TAT **GG**C ACG AT**T** G	Asp
E.coli_GyA87C5	87	85(GTC)/89(ATT)	GCG GT**C** TAT **GC**C ACG AT**T** G	His
E.coli_GyA87A6	87	85(GTT)/89(ATT)	GCG GT**T** TAT **GA**C ACG AT**T** G	Asn
E.coli_GyA87T6	87	85(GTT)/89(ATT)	GCG GT**T** TAT **GT**C ACG AT**T** G	Tyr
E.coli_GyA87G6	87	85(GTT)/89(ATT)	GCG GT**T** TAT **GG**C ACG AT**T** G	Asp
E.coli_GyA87C6	87	85(GTT)/89(ATT)	GCG GT**T** TAT **GC**C ACG AT**T** G	His
E.coli_GyA87A7	87	85(GTC)/89(ATC)	GCG GT**C** TAT **GA**C ACG AT**C** G	Asn
E.coli_GyA87T7	87	85(GTC)/89(ATC)	GCG GT**C** TAT **GT**C ACG AT**C** G	Tyr
E.coli_GyA87G7	87	85(GTC)/89(ATC)	GCG GT**C** TAT **GG**C ACG AT**C** G	Asp
E.coli_GyA87C7	87	85(GTC)/89(ATC)	GCG GT**C** TAT **GC**C ACG AT**C** G	His
E.coli_GyA87A8	87	85(GTC)/89(ATT)	GCG GT**T** TAT **GA**C ACG AT**C** G	Asn
E.coli_GyA87T8	87	85(GTC)/89(ATT)	GCG GT**T** TAT **GT**C ACG AT**C** G	Tyr
E.coli_GyA87G8	87	85(GTC)/89(ATT)	GCG GT**T** TAT **GG**C ACG AT**C** G	Asp
E.coli_GyA87C8	87	85(GTC)/89(ATT)	GCG GT**T** TAT **GC**C ACG AT**C** G	His
E.coli_GyA87AU2	87	85(GTI)/89(ATI)	GCG GT**I** TAT **GA**C ACG AT**I** G	Asn
E.coli_GyA87TU2	87	85(GTI)/89(ATI)	GCG GT**I** TAT **GT**C ACG AT**I** G	Tyr
E.coli_GyA87GU2	87	85(GTI)/89(ATI)	GCG GT**I** TAT **GG**C ACG AT**I** G	Asp
E.coli_GyA87CU2	87	85(GTI)/89(ATI)	GCG GT**I** TAT **GC**C ACG AT**I** G	His

Capture probes directed against amino acid position 83 and 87 of GyrA with consideration of nucleotide variations at position 85 and 89.

All the probes are 19mer and with the SNPs position almost in the middle.

Bold letter indicate the SNPs positions and underline letter indicate the positions with variation.

For position 83 two sets of probes (eight probes) and for position 87 eight sets of probes (32 probes) were designed, which four sets were directed against the first position of the triplet code, while the other four sets are directed against the second position of the triplet code.

Both for position 83 and 87 were universal probes (for position 83 one set and for position 87 two sets) designed, which had inosine at the positions with variations.

[0039] According to a preferred embodiment the array may contain at least one additional set of capture probes, derived from the parC gene of *E.coli.* In fact, the topoisomerase IV is the secondary target for quinolone in the case of *E. coli.* The point mutation of the A subunit of this enzyme, which is encoded by parC gene, is the main cause for the resistance. Three amino acid positions, i.e. residues, 80, 84 and 87, have been chosen as locations for the detection. Frequent mutations at position 80 include Ser to Ile or Arg. Common mutations at position 84 include Glu to Lys or Gly.

[0040] As for the set of probes directed to the gyrA mutations also in this set of probes (directed to the parC gene), the capture probes on the micro-array comprise the sequence R_1-(Y)-

R_2 and may be provided in either of one or two or three sets on the array. In one embodiment, the inventive micro-array contains, apart from the capture probes directed to the gyrA gene, capture probes directed to the parC gene. The sequences R_1 and R_2, which may be of a length of from about 5 to about 20 nucleotides each, are derived from the sequences of the parC gene of *E.coli* adjacent to the triplet encoding the amino acid at position 80 in the parC polypeptide. In a second and third set of capture probes, respectively, the sequences R_1 and R_2, which may exhibit the same length as indicated above, are derived from the sequence of the gyrA gene of *E.coli* adjacent to the triplet encoding the amino acid no. 84 or 87 in the parC polypeptide.

[0041] In a next step it will be determined at which location on the array binding occurred, which is generally achieved by detecting the label that has been attached to/incorporated in the target-DNA prior to the hybridization step, or by performing a labelling reaction on the array. So called "direct labels" are detectable labels that are directly attached to or incorporated into the target (sample) nucleic acid prior to hybridization. In contrast, so called "indirect labels" are joined to the hybrid duplex after hybridization. The indirect label may also be attached to a binding moiety that has been attached to the target nucleic acid prior to the hybridization. For a detailed review of methods of labelling nucleic acids and detecting labelled hybridized nucleic acids see Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 24: Hybridization With Nucleic Acid Probes, P. Tijssen, ed. Elsevier, N.Y., (1993)).

[0042] Also, the capture probes present on the array may contain a label at their 3'-end. After binding of the target DNA, the DNA/DNA hybrids are then cleaved with a particular enzyme thus releasing the label from those capture probes, where the target DNA had bound. Therefore, in this embodiment the decrease in signal is representative of the presence of a given nucleotide sequence in the gyrA gene.

[0043] Means of detecting labeled target nucleic acids hybridized to probes are well-known to those skilled in the art.

[0044] Two types of divergent results may be obtained.

[0045] On the one hand it may be noted that at the location representing the triplet of the native, *i.e.* quinolone sensitive strain, binding occurred, which will be indicative of a quinolone sensitive strain.

[0046] On the other hand it may also be observed that binding occurs on a location representing a triplet different from the native one. In this case, it should be first determined

whether the change in the triplet has led to a change in the respective amino acid, either in one or two of the positions, preferably from serine to leucine (at position 83) and/or aspartate to asparagine or tyrosine at position 87.

[0047] This step of evaluating whether the mutation has led to a change of an amino acids may also be obviated by spotting only such kind of mutations on the array which also lead to a change of an amino acid (cf. wobble hypothesis). However, proceeding accordingly harbors the disadvantage that in such a case no signal will be obtained for this position, wherein the skilled person has to rely solely on the positive control to be ascertained that the experiment really worked. For this reason, a micro-array harboring all of the possible mutations of the respective triplets in the corresponding sets is preferred for use in the present method.

[0048] The present method, therefore, provides a reliable and rapid means for determining, whether or not a given biological sample contains an *E.coli* strain, having developed resistance against quinolones. Since the assay is easy to carry out an attending physician may quickly obtain the required information and may apply an appropriate regimen

EXAMPLE

Micro-array for detecting quinolone-resistant *Escherichia coli*

1. Biological Material

[0049] A total of 29 quinolone-resistant *E. coli* clinical isolates from four different hospitals in Germany and one quinolone-sensitive clinical isolate were used in this study. These strains have been isolated from urine (n=20), swab (from the lower leg n=1, foot n=1, throat n=2, groin n=1, abscess n=1, unknown n=1) (n=7), secretion (tracheal secretion n=1, bronchial secretion n=1) (n=2) and blood (n=1) of patients. The susceptibility of the strains against quinolone was determined either by using Ciprofloxacin (n = 23) alone or by using both Ciprofloxacin and Levofloxacin (n = 7). Genomic DNA was isolated using QIAamp DNA Mini Kit (Qiagen) according to protocol provided by the manufacturer.

2. DNA Sequencing and Amplification

[0050] The gyrA gene of some isolates was sequenced by amplifying the gene from the isolates using primers that yielded overlapping fragments. The sequencing of 5 isolates gave the preliminary result that apart from a variety of different mutations all of them had a common mutation at position 83 and 87 in the gyrA gene.

[0051] In order to verify the initial finding the region in the gyrA gene, a 417 bp long fragment from nucleotide position 119 to 535 around these positions was amplified by using the following primers:

[0052] forward primer Gyr_coli_F1 (5'-ccatacctacggcgataccg-3'), and

[0053] reverse primer Gyr_coli_R1 (5'-gcctgaagccggtacaccgt-3').

[0054] The PCR mixtures (50 µl) included about 80 ng template of genomic DNA of *E. coli*), 0.4 pM (pmol/L) of each primer, 0.25 mM dNTPs (desoxyribonucleoside-5'-triphosphate), 1.5 mM Mg^{2+} (mmol/L) and 2.5 U Taq Polymerase (Eppendorf). The PCRs were performed in a thermocycler (Eppendorf) using following parameters: 94°C 5 min; 94°C 1 min, 52°C 1 min, 72°C 1 min for 30 cycles; final elongation 72°C 10 min. The amplified fragment, which was purified using QIAquick PCR purification kit (Qiagen) according to the manual provided by the manufacture, was used for direct sequencing. The sequencing was done using the same primer pairs with big-dye terminator cycle sequencing kit (Applied Biosystem) and Prism™ 377A-DNA-sequencer (Applied Biosystems).

[0055] For all the investigated resistant strains it was noted that they exhibited a mutation at positions 83 and 87, which were at position 83 from serine (codon TCG) to leucine (codon TTG) (n = 28) and at position 87 from aspartate (codon GAC) to asparagine (codon AAC)(n = 27) or to tyrosine (codon TAC) (n = 1) or to glycine (codon GGC) (n=1). It was also noted that these 30 isolates belong to two variants. The one variant (n = 27) had at position 85 codon GTT (Val), at position 91 codon CGT (Arg) and at position 100 codon TAC (Tyr). The other variant (n = 3) had at position 85 codon GTC (Val), at position 91 codon CGC (Arg) and at position 100 codon TAT (Tyr).

Table 2

Position 83	Position 85	Position 87	Position 89	Position 100*	Number of Isolate	Phenotype
TCG (Ser)	GT**T**	GAC (Asp)	CG**T**	TA**C**	1	sensitive
TTG (Leu)	GT**C**	AAC (Asn)	CG**C**	TA**T**	3	resistant
TTG (Leu)	GT**T**	AAC (Asn)	CG**T**	TA**C**	24	resistant
TTG (Leu)	GT**T**	TAC (Tyr)	CG**T**	TA**C**	1	resistant
TCG (Ser)	GT**T**	G**G**C (Gly)	CG**T**	TA**C**	1	resistant

Bold letter indicate the nucleotide change, which lead to amino acid substitution (mutation) and underline letter indicate nucleotide change, which have no effect on amino acid (variation). Asterisk indicate that the sequences of this position were determined by sequencing;

Genotype of 30 clinical isolates determined using micro-array. The phenotype was determined by using Ciprofloxacin alone or by using both Ciprofloxacin and Levofloxacin.

3. Array Fabrication

[0056] The possibility of using a micro-array to enable high through-put analysis was evaluated. Using Microgrid II (Biorobotics), 20 µM or 40 µM oligonucleotide capture probes (cf. table I), which have been dissolved in 50% (Vol./Vol.) in DMSO, were spotted on poly-L-lysine slides (Sigma) in two subarrays. Each slide was also spotted with spotting control (5'-Cy5-tctagacagccactcata-3') (Cy5 labeled oligonucleotide), hybridization control (5'-gattggacgagtcaggagc-3') oligonucleotide with unrelated sequence referring to *gyrA*, whose Cy5 labeled complement oligonucleotide would be included in hybridization solution) and process control (5'-taatgggtaaataccatcc-3') oligonucleotide with consensus sequence of *gyrA*). After spotting, the slides were irradiated with UV light at 120 mJ/m^2 using UV crosslinker (Biometra) and blocked using an aqueous blocking solution (0.18 M succinic anhydride in methyl-pyrrolidinone / 44 mM Na-borate pH 8.0) for 10 min, followed by rinse in distilled water and subsequently in 100% ethanol, and finally dried for about 10 min.

4. Amplification and Labeling

[0057] An amplification of target DNA an concurrent labeling was performed using the following primers:

[0058] Forward primer GyrA_coli_F3 (5'-acgtactaggcaatgactgg-3'); and

[0059] reverse primer GyrA_coli_R3 (5'-agagtcgccgtcgatggaac-3').

[0060] The 50 µl PCR mixture included about 80 ng template (genomic DNA of *E. coli*), 0.4 pM (pmol/L) of each primer, 0.1 mM dATP, 0.1 mM dGTP, 0.1 mM dTTP, 0.06 mM dCTP, 0.04 mM Cy5-dCTP, 1.5 mM Mg^{2+} and 2.5 U Taq polymerase (Eppendorf). The PCRs were performed in a thermocycle (Eppendorf) using the same parameters as described before. The amplified 189 bp fragment, which was purified using QIAquick PCR purification kit (Qiagen) according to the manual provided by the manufacture, was used for hybridization.

5. Hybridization, Washing and Scanning

[0061] The purified amplicon in 40 µl hybridization solution (6 x SSPE) plus 0.1 pmol Cy5 labeled DNA for the hybridization control were incubated on the slides prepared as above at 45°C over night in a hybridization chamber (Corning) or alternatively three hours in a hybridization station. For manual hybridization 4 pmol target DNA was used, while for hybridization in a hybridization station 0.78 pmol target DNA was used. After hybridization, the slides were

washed with 2 X SSC, 0.1% (w/v) SDS for 15 min, with 0.2 X SSC for 3 min at room temperature and dried with N_2. For detection slides were scanned using Array Scanner GMS 418 (Affymetrix) at Cy5 channel. The images were analyzed using ImaGene (BioDiscovery) and saved as plain-text file as raw data.

[0062] The results obtained by means of the array correspond to the results achieved by means of sequencing. The present method, therefore, provides an efficient means to rapidly, *i.e.* within about there to 5 hours, determine the presence or absence of a quinolone sensitive or resistant strain.

6. Hybridisation of labelled target DNA of clinical isolate on microarray

[0063] To further demonstrate the applicablity of the claimed invention, a micro-array was designed to evaluate position 83. An array was prepared with the capture probes depicted in Table 1 for position 83. The layout of the probes is depicted in Figure 1(A). Two variants were used: GTC at position 85 for variant 1, and GTT at position 85 for variant 2. The results are depicted in Figure 1. Panel (B) depicts a quinolone-senstive *E.coli*, while panels (C) and (D) show two different *E. coli* variants with quinolone resistance.